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The *Tetrahymena* Argonaute-Binding Protein Giw1p Directs a Mature Argonaute-siRNA Complex to the Nucleus

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SUMMARY

Emerging evidence suggests that RNA interference (RNAi)-related processes act both in the cytoplasm and in the nucleus. However, the process by which the RNAi machinery is transported into the nucleus remains poorly understood. The *Tetrahymena* Argonaute protein Twi1p localizes to the nucleus and is crucial for small RNA-directed programmed DNA elimination. In this study, we identify Giw1p, which binds to Twi1p and is required for its nuclear localization. Furthermore, the endoribonuclease (Slicer) activity of Twi1p plays a vital role in the removal of one of the two strands of Twi1p-associated small interfering RNAs (siRNAs), leading to a functionally mature Twi1p-siRNA complex. Slicer activity is also shown to be required for nuclear localization of Twi1p and for its association with Giw1p. These results suggest that Giw1p senses the state of Twi1p-associated siRNAs and selectively transports the mature Twi1p-siRNA complex into the nucleus.

INTRODUCTION

Argonaute family proteins bind to small RNAs (~20–30 nt) and are integral players in all known RNA interference (RNAi)-related gene-regulatory pathways (reviewed in Tolia and Joshua-Tor, 2007). Many Argonaute proteins act in the cytoplasm, where they induce posttranscriptional gene silencing. Recent evidence suggests that Argonaute proteins also act in the nucleus.

In mammals, the Argonaute proteins Ago1 and Ago2 mediate transcriptional silencing (Janowski et al., 2006; Kim et al., 2006) and Ago2 localizes to the nucleus in an Importin 8-dependent manner (Weinmann et al., 2009). Another Argonaute protein, MIWI2, localizes to the nucleus in fetal mouse testes and is required for DNA-methylation-mediated retrotransposon silencing (Aravin et al., 2008; Kuramochi-Miyagawa et al., 2008). The *Drosophila* Argonaute protein Piwi localizes to nuclei of nurse and follicle cells in the ovary (Cox et al., 2000; Brennecke et al., 2007) and plays a role in transcriptional gene silencing (Pal-Bhadra et al., 2002). In *Arabidopsis*, the nuclear-localizing Argonaute proteins AGO4 and AGO6 are involved in RNA-directed DNA methylation (Li et al., 2006; Pontes et al., 2006; Zheng et al., 2007). In the fission yeast *Schizosaccharomyces pombe*, the Argonaute protein Ago1 is involved in both transcriptional and posttranscriptional gene silencing (Volpe et al., 2002; Sigova et al., 2004) and localizes to both the cytoplasm and the nucleus (Noma et al., 2004).

Two recent studies indicate that nuclear import of some Argonaute proteins is dependent on small RNAs. The *Caenorhabditis elegans* Argonaute protein NRDE-3 needs to associate with a small interfering RNA (siRNA) to localize to the nucleus (Guang et al., 2008). In mice, nuclear localization of MIWI2 requires MILI, which is essential for the production of Piwi-associated (pi) RNAs that bind MIWI2 (Aravin et al., 2008). These studies suggest that some mechanism distinguishes between free Argonaute proteins and those complexed with small RNAs, transporting only the latter into the nucleus. However, little is known about how small RNAs regulate the nuclear localization of Argonaute proteins.

The ciliated protozoan *Tetrahymena thermophila* provides an extreme example of a nuclear-acting Argonaute protein. The Argonaute protein Twi1p plays an essential role in programmed DNA elimination (Mochizuki et al., 2002), which is evolutionarily related to RNAi-directed heterochromatin formation in other eukaryotes (reviewed in Malone and Hannon, 2009). *Tetrahymena* possesses a germline micronucleus and a somatic macronucleus in a single cell. The micronucleus produces both new micronuclei and new macronuclei during sexual reproduction. During macronuclear development, ~6000 different internal eliminated sequences (IESs) are defined by ~28–29 nt siRNAs, termed scan RNAs (scn) RNAs (Mochizuki et al., 2002; Yao et al., 2003; Lee and Collins, 2006), and removed. scnRNAs are processed from bidirectionally transcribed noncoding RNAs (Chalker and Yao, 2001) by the Dicer-like protein Dcl1p in the

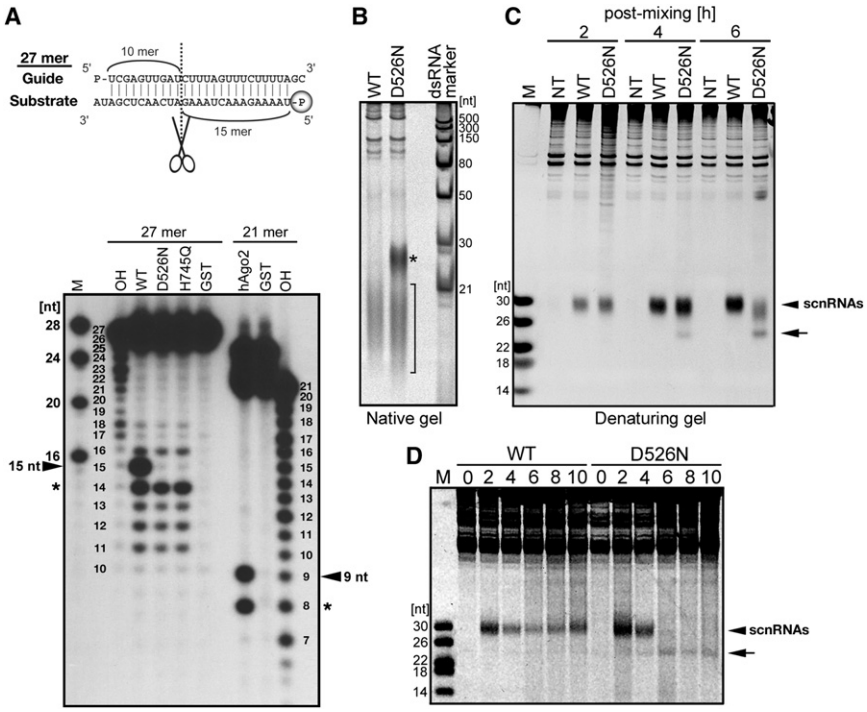

Figure 1. Twi1p Has Slicer Activity that Is Important for Passenger-Strand Removal of scnRNAs

(A) Wild-type (GST-Twi1p-WT) or Slicer-dead (GST-Twi1p-D526N, GST-Twi1p-H725Q) recombinant Twi1p, or GST was incubated with a 27 nt guide-strand RNA and then with 5′-end-labeled substrate RNA. Wild-type, but not Slicer-dead, Twi1p cleaved the substrates between residues base paired to nucleotides 10 and 11 of the guide strands, thus producing 15 nt labeled products. As a control, recombinant human Ago2 (hAgo2) was incubated with 21 nt guide and target RNAs, resulting in 9 nt labeled products. 20 and 24 nt RNA oligos (M) and partially alkaline hydrolysed substrate RNAs (OH) were 5′ end labeled and used as position markers. Asterisks indicate byproducts that have also been detected in other in vitro assays.

(B) scnRNAs coimmunoprecipitated with FLAG-HA-Twi1p-WT (WT) and FLAG-HA-Twi1p-D526N (D526N) at 4 hr postmixing were separated in a native gel and stained by GelRed. Double- and single-stranded scnRNAs are marked with an asterisk and a bracket, respectively.

(C) scnRNAs coimmunoprecipitated with FLAG-HA-Twi1p-WT (WT) or FLAG-HA-Twi1p-D526N (D526N) at 2, 4, and 6 hr postmixing were separated in a denaturing gel and stained by GelRed. Nontagged wild-type strains (NT) were used as a negative control. The position of scnRNAs and ~24 nt RNAs are marked by an arrowhead and an arrow, respectively. M: single-stranded RNA markers.

(D) Total RNA from FLAG-HA-*TWI1*-WT (WT) and FLAG-HA-*TWI1*-D526N (D526N) at 0, 2, 4, 6, 8, and 10 hr postmixing was separated in a denaturing gel and stained by GelRed. The position of scnRNAs and ~24 nt RNAs are marked by an arrowhead and an arrow, respectively.

See also Figure S1.

micronucleus (Malone et al., 2005; Mochizuki and Gorovsky, 2005) and complex with Twi1p in the cytoplasm (Mochizuki and Gorovsky, 2004). The Twi1p-scnRNA complex is then transported into the parental macronucleus, where it has been proposed that IES-specific scnRNAs are enriched through selective degradation of scnRNAs that are complementary to the macronuclear DNA, probably by recognizing nascent transcripts (Mochizuki et al., 2002; Aronica et al., 2008). Finally, Twi1p-scnRNA complexes move into the developing macronucleus, where they induce the formation of heterochromatin, leading to DNA elimination (Mochizuki et al., 2002; Taverna et al., 2002; Liu et al., 2007).

These dynamic changes in the localization of Twi1p are believed to be essential for the conserved small RNA-directed heterochromatin-formation process, providing an attractive model for the study of how Argonaute-small RNA complexes are localized and how their localizations influence their functions in eukaryotes. Here, we report that nuclear localization of Twi1p is regulated by the Twi1p-binding protein Giw1p, which senses the state of siRNAs associated with Twi1p. This mechanism enables *Tetrahymena* to transport only a functionally mature Argonaute-siRNA complex into the nucleus.

RESULTS

Twi1p Has DDH-Motif-Dependent Slicer Activity

Some Argonaute proteins have endoribonuclease (Slicer) activity responsible for cutting RNAs with sequences complementary to those of their small RNA cargos. Slicer activity is provided by the evolutionarily conserved Piwi domain, when it contains a conserved catalytic core composed of an Asp-Asp-His (DDH) motif (reviewed in Tolia and Joshua-Tor, 2007). Comparison of the Piwi domains of the *Tetrahymena* Argonaute protein Twi1p and other Argonaute proteins possessing Slicer activity (Figure S1A, available online; Couvillion et al., 2009) revealed that Twi1p contains a DDH motif (Asp526-Asp596-His745), suggesting that it may have Slicer activity.

Slicer activity of Twi1p was analyzed with the use of recombinant Twi1p expressed in *E. coli* as a GST fusion protein. GST-Twi1p was incubated with 27 nt "guide" RNA to form RISC-like ribonucleoprotein complexes. These complexes were then incubated with a 5′ end radio-labeled 27 nt "substrate" RNA whose 3′ 25 nt were complementary to the guide RNA. As a positive control, recombinant human Ago2 fused to GST (Rivas et al., 2005) was prepared, complexed with 21 nt guide RNA, and incubated with a 5′ end radio-labeled 21 nt substrate RNA whose 3′ 19 nt were complementary to the guide RNA. The cleaved product was observed by denaturing gel electrophoresis followed by autoradiography (Figure 1A). If the GST-Twi1p cleaves the substrate in a manner similar to that of other Argonaute proteins with Slicer activity, which cleave the bond between residues base-paired to nucleotides 10 and 11 of the guide strand (reviewed in Tolia and Joshua-Tor, 2007), the radio-labeled cleavage product should be 15 nt long (see schematic drawing in Figure 1A). Indeed, a 15 nt RNA species was detected in the

GST-Twi1p sample (Figure 1A, 27-mer, WT), but not when GST was used alone (Figure 1A, 27-mer, GST). Similarly, radiolabeled cleavage product indicating cutting between residues base paired to nucleotides 10 and 11 of the guide strand (9 nt) was detected with the use of GST-hAgo2 and 21 nt RNAs (Figure 1A, 21-mer, GST-hAgo2). We conclude that Twi1p possesses Slicer activity.

Cleavage of substrate RNA by GST-Twi1p in our assay was less efficient than that by some other Argonautes in similar assays (Matranga et al., 2005; Miyoshi et al., 2005; Rand et al., 2005). A pre-steady-state kinetics analysis of substrate RNA cleavage (Förstemann et al., 2007) suggested that only ~0.4% of the recombinant GST-Twi1p was active (Figure S1B). The inefficient substrate cleavage by GST-Twi1p may be caused either by enzymatic inactivity of the majority of GST-Twi1p or by inefficient complex formation between Twi1p-guide-strand scnRNA in vitro.

For determining whether the DDH motif is involved in the Slicer activity of Twi1p, mutants were created in which either the first aspartic acid of the motif was replaced by asparagine (Twi1p-D526N) or the last histidine of the motif was replaced by glutamine (Twi1p-H754Q) (Figure S1A). Twi1p-D526N or Twi1p-H745Q fused to GST was analyzed as described above. As shown in Figure 1A, neither mutated enzyme produced a detectable 15 nt cleavage product, suggesting that the conserved motif is required for the Slicer activity of Twi1p. These mutated Twi1p proteins are referred to as Slicer-dead Twi1p.

Slicer Activity of Twi1p Is Important for scnRNA Passenger-Strand Removal In Vivo

In vivo, Twi1p associates with ~28–29 nt siRNAs, named scnRNAs (Mochizuki and Gorovsky, 2004). Given that scnRNAs are processed from double-stranded noncoding RNA by the Dicer-like protein Dcl1p (Malone et al., 2005; Mochizuki and Gorovsky, 2005), there must be a mechanism responsible for making scnRNAs single stranded. Because the Slicer activities of several Argonaute proteins are involved in the removal of one of the two small RNA strands (passenger strand) in other organisms (Matranga et al., 2005; Miyoshi et al., 2005; Rand et al., 2005; Leuschner et al., 2006; Maiti et al., 2007; Steiner et al., 2009), we tested whether the Slicer activity of Twi1p was involved in scnRNA passenger-strand removal in vivo.

We constructed *Tetrahymena* strains whose *TWI1* loci in the polyploid macronucleus were completely replaced by a mutant construct (*FLAG-HA-TWI1-D526N*) encoding Slicer-dead Twi1p-D526N tagged with FLAG-HA (Figures S1C and S1E). *FLAG-HA-TWI1-WT*, expressing wild-type Twi1p tagged with FLAG-HA (Figures S1C and S1D), was also used. Two *FLAG-HA-TWI1-WT* strains of different mating types produced viable sexual progeny (Figure S1M), indicating that the FLAG-HA tag does not disturb the essential function (Mochizuki et al., 2002) of Twi1p. Comparable amounts of FLAG-HA-Twi1p-WT and FLAG-HA-Twi1p-D526N were detected by Western blotting (Figure S1H), indicating that the Slicer activity is not required for the accumulation of Twi1p protein.

scnRNAs coimmunoprecipitated with FLAG-HA-Twi1p-D526N or FLAG-HA-Twi1p-WT from cells at an early stage (4 hr postmixing) of conjugation were separated in native gel and stained with GelRed (Figure 1B). More than half of the FLAG-HA-Twi1p-D526N-associated scnRNAs detected by the staining migrated at positions corresponding to double-stranded ~28 nt RNA (Figure 1B, asterisk). The remaining scnRNAs from FLAG-HA-Twi1p-D526N and all scnRNAs from FLAG-HA-Twi1p-WT migrate as a smear (Figure 1B, bracket). We believe that this smear signal is attributable to the extensive sequence heterogeneity of the scnRNAs, which are believed to be transcribed from the whole micronuclear genome (Mochizuki et al., 2002). Consistent with this, by northern hybridization, two different 28 nt oligo DNA probes complementary to different specific scnRNAs sequences detected distinct bands within the smear region (Figure S1N). Because of their small size and AT richness, a fraction of scnRNAs could dissociate during experimental handling, and this analysis likely underestimates the amount of double-stranded scnRNA associated with FLAG-HA-Twi1p-D526N. In contrast, none of the scnRNA associated with FLAG-HA-Twi1p-WT migrated to the position on the gel corresponding to double-stranded scnRNA (Figure 1B, WT). Denaturing gel analysis of these scnRNAs indicated that similar amounts of scnRNA were associated with FLAG-HA-Twi1p-D526N and FLAG-HA-Twi1p-WT at 2 hr and 4 hr postmixing (Figure 1C). We conclude that the Slicer activity of Twi1p plays an important, possibly essential, role in the scnRNA passenger-strand removal in vivo.

Slicer Activity of Twi1p Is Required for Stable Accumulation of scnRNA

We analyzed the expression of scnRNAs in the absence of the Slicer activity of Twi1p. *FLAG-HA-TWI1-D526N* strains expressed levels of scnRNAs similar to those of *FLAG-HA-TWI1-WT* strains in the early stages of conjugation (Figure 1D, 2–4 hr postmixing). However, in the *FLAG-HA-TWI1-D526N* strains, the amount of scnRNA was greatly reduced at the mid stage of conjugation (Figure 1D, 6 hr) and became undetectable at later stages of conjugation (Figure 1D, 8–10 hr). Thus, Slicer activity of Twi1p is not required for production of scnRNAs, but it is required for their stable accumulation. An exonuclease likely degrades the double-stranded scnRNAs complexed with Slicer-dead Twi1p, as the scnRNAs associated with FLAG-HA-Twi1p-D526N become gradually shorter and less abundant (Figure 1C, 4–6 hr). We previously reported that the RNA methyltransferase Hen1p methylates only single-stranded scnRNAs to protect them from degradation (Kurth and Mochizuki, 2009). The mid-stage disappearance of scnRNAs in Slicer-dead *TWI1* cells could be a result of a lack of methylation of double-stranded scnRNAs.

Approximately 24 nt of RNA bound to FLAG-HA-Twi1p-D526N in the mid stages of conjugation (4–6 hr postmixing) (Figure 1C, arrow) and accumulated (Figure 1D, arrow). Northern blot analysis demonstrated that ~28–29 nt scnRNAs, but not the ~24 nt RNAs, hybridize to a Tlr1-1 oligo DNA probe, which is complementary to a subset of scnRNAs derived from repeated Tlr1 IES elements (Figure S1O). Therefore, the ~24 nt RNAs probably are not degradation products of scnRNAs but are likely constitutively expressed ~23–24 nt siRNAs (Lee and Collins, 2006). These ~23–24 nt siRNAs might misassociate with Twi1p and therefore be stabilized when scnRNAs are reduced.

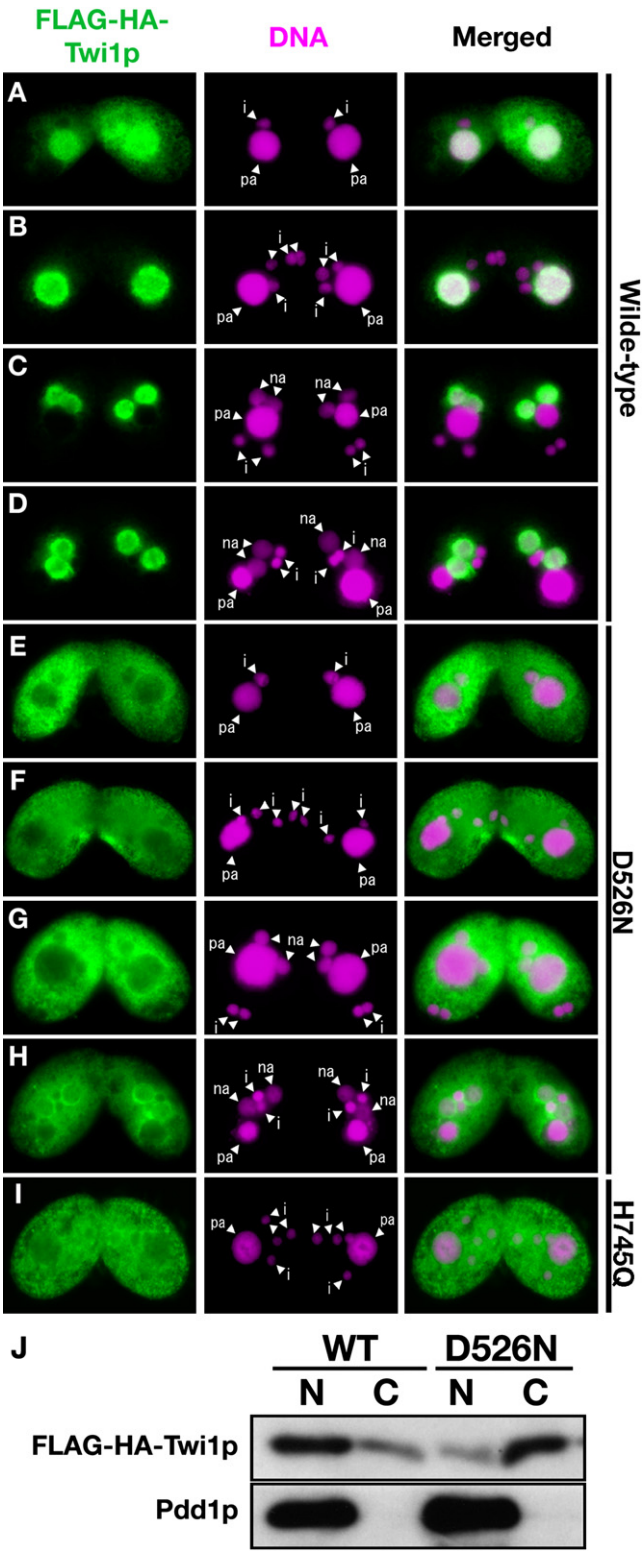

Figure 2. Slicer Activity of Twi1p Is Required for the Macronuclear Localization of Twi1p

(A–I) FLAG-HA-Twi1p-WT (Wild-type, A–D), FLAG-HA-Twi1p-D526N (D526N, E–H) and FLAG-HA-Twi1p-H745Q (H745Q, I) were localized by an anti-HA antibody (green). DNA was stained by DAPI (purple). The micronuclei (i), the parental macronuclei (pa), and the newly developed macronuclei (na) are marked.

(J) The nuclear (N) and the cytoplasmic (C) fractions from FLAG-HA-TWI1-WT (WT) or FLAG-HA-TWI1-D526N (D526N) at 9 hr postmixing (corresponding to the stages shown in D and H) were analyzed by Western blotting. FLAG-HA-Twi1p and Pdd1p were detected by an anti-HA and an anti-Pdd1p antibody, respectively.

See also Figure S2.

Slicer Activity of Twi1p Is Required for Twi1p Nuclear Localization

The localization of FLAG-HA-Twi1p-WT and FLAG-HA-Twi1p-D526N was analyzed by indirect immunofluorescence staining with the use of an anti-HA antibody. FLAG-HA-Twi1p-WT localized to both the cytoplasm and the parental macronucleus during early stages of conjugation (Figure 2A). During mid stages, it localized almost exclusively to the parental macronucleus (Figure 2B). In the later stages of conjugation, FLAG-HA-Twi1p-WT disappeared from the parental macronucleus and appeared in the newly developing macronucleus (Figures 2C and 2D). This localization pattern was indistinguishable from that of nontagged wild-type Twi1p detected with an anti-Twi1p antibody (see below), indicating that the presence of the FLAG-HA tag did not disturb the localization of Twi1p.

In contrast, FLAG-HA-Twi1p-D526N was detected in the cytoplasm throughout conjugation and did not accumulate in the parental macronucleus (Figures 2E–2G). In the late stages of conjugation, FLAG-HA-Twi1p-D526N accumulated at the periphery of the newly developing macronuclei but was still seen mostly in the cytoplasm (Figure 2H). These results were confirmed by analyzing nuclear and cytoplasmic fractions via Western blotting (Figure 2J). Though FLAG-HA-Twi1p-WT was detected mainly in the nuclear fraction, FLAG-HA-Twi1p-D526N was detected mainly in the cytoplasmic fraction. In contrast, the macronuclear protein Pdd1p (Coyne et al., 1999) was detected in the nuclear fraction in both FLAG-HA-*TWI1*-WT and FLAG-HA-*TWI1*-D526N strains. The other Slicer-dead Twi1p mutant (FLAG-HA-Twi1p-H745Q; Figures S1F and S1G) also localized to the cytoplasm (Figure 2I, Figure S2). These results indicate that Slicer activity is required for the Twi1p nuclear localization.

Given that comparable levels of scnRNAs accumulate in both wild-type and Slicer-dead FLAG-HA-*TWI1*-D526N strains at 4 hr postmixing (Figure 1D), at which point wild-type Twi1p was already localized to the parental macronucleus (Figure 2B), the mislocalization of Slicer-dead Twi1p was not likely due to the nucleolytic shortening or reduction of scnRNAs in the mutants; instead, it is probably directly caused by defective passenger-strand removal of scnRNAs.

Two possible mechanisms could explain Slicer-dependent Twi1p nuclear localization. The first suggests that Twi1p complexed with single-stranded scnRNA is anchored in the nucleus through an interaction between scnRNA and nascent macronuclear noncoding transcripts (Aronica et al., 2008). However, this anchoring cannot fully explain the nuclear localization of Twi1p, because *EMA1* KO strains, in which the scnRNA-noncoding RNA interaction is impaired, show normal Twi1p macronuclear localization (Aronica et al., 2008). The second and more likely

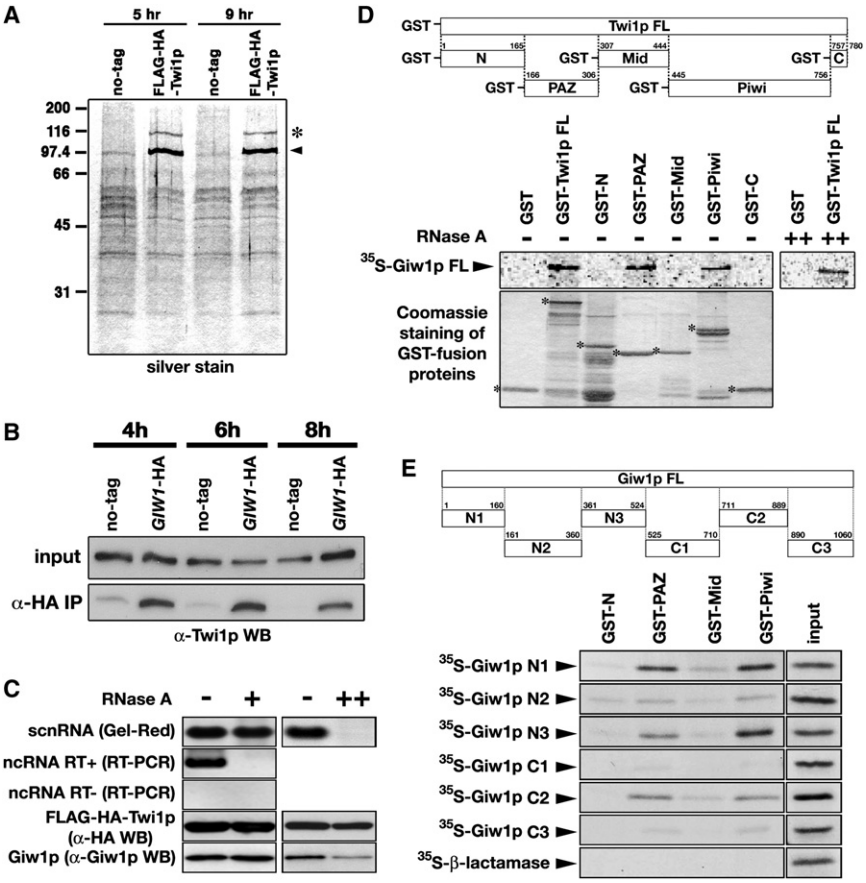

Figure 3. Giw1p Interacts with Twi1p

(A) Proteins copurified with FLAG-HA-Twi1p at 5 and 9 hr postmixing were separated by SDS-PAGE and visualized by silver staining. As a control, wild-type (no-tag) strains were processed in parallel. Positions of FLAG-HA-Twi1p-WT and ~115 kDa protein are marked by an arrowhead and an asterisk, respectively.

(B) Cell lysates were prepared from wild-type (no-tag) or *GIW1-HA* strains at 4, 6, and 8 hr postmixing, and immunoprecipitation was performed with an anti-HA antibody. Twi1p in the cell lysate (input) and immunoprecipitated samples (α-HA IP) were detected by Western blot with the use of an anti-Twi1p antibody.

(C) Cell lysate from FLAG-HA-*TWI1*-WT strains at 4 hr postmixing was incubated with 20 pg/mL (+), 100μg/mL (++), or without (−) RNase A, and FLAG-HA-Twi1p-containing complexes were immunoprecipitated with the use of an anti-FLAG antibody. Coimmunoprecipitated scnRNA was stained by GelRed. Coprecipitated noncoding RNA from the macronuclear R locus was analyzed by RT-PCR with (ncRNA RT+) or without (ncRNA RT−) reverse transcriptase. Precipitated FLAG-HA-Twi1p and Giw1p were detected by Western blotting with the use of anti-FLAG and anti-Giw1p antibodies, respectively.

(D) GST pull-down assays were performed with the use of GST, full-length Twi1p fused to GST (GST-Twi1pFL), or parts of Twi1p (N-terminal, PAZ, mid, or Piwi domains) fused to GST, and ^{35}S-labeled full-length Giw1p (^{35}S-Giw1p FL) with 100 μg/mL (++) or without (−) RNaseA. The precipitated proteins were separated by SDS-PAGE, and ^{35}S-Giw1p FL was detected by phosphorimager. One-fourth of GST and GST-Twi1p recombinant proteins (asterisks) used for the assay were stained by Coomassie blue.

(E) Different segments of ^{35}S-labeled recombinant Giw1p were expressed and pulled down with GST or parts of Twi1p (N-terminal, PAZ, mid, or Piwi domains) fused to GST. The precipitated proteins were separated by SDS-PAGE, and the ^{35}S-labeled Giw1p segments were detected by autoradiography. ^{35}S-labeled β-lactamase was used as a negative control. For comparison of the relative intensity of bands between the experiments, the precipitated proteins were exposed to X-ray films for the same period and processed equally. ^{35}S-labeled Giw1p segments used for the pull-down assay were analyzed in a different gel (input). See also Figure S3.

possibility is that an active macronuclear import mechanism specifically recognizes the complex formed between Twi1p and single-stranded scnRNA.

Giw1p is a Twi1p-Associated Protein

None of the previously identified Twi1p-associated proteins are required for macronuclear localization of Twi1p (Aronica et al., 2008; Bednenko et al., 2009). For the identification of Twi1p-associated proteins involved in the macronuclear localization of Twi1p, FLAG-HA-Twi1p-containing complexes were isolated with lysis and washing conditions that were milder (see Experimental Procedures) than those used in the previous studies. Immunoprecipitated samples from cells at the mid (5 hr postmixing) and the late (9 hr) stages of conjugation were separated by SDS-PAGE and analyzed by silver staining (Figure 3A). In addition to a band corresponding to FLAG-HA-Twi1p, a previously unidentified ~115 kDa protein was detected in *FLAG-HA-TWI1* strains but not in nontagged, wild-type strains. In this study, the three previously identified Twi1p-associated proteins (Ema1p [211 kDa], CnjBp [200 kDa], and Wag1p [123 kDa]) were undetectable by silver staining, although Ema1p and Wag1p were weakly detectable by Western blotting (Figure S3A), most likely due to the milder lysis conditions employed. The milder lysis procedure used here solubilizes mainly cytoplasmic components, including the 115kDa protein (see below), whereas all three previously identified Twi1p-associated proteins localize mainly to nuclei (Aronica et al., 2008; Bednenko et al., 2009) and require harsher lysis conditions to be observed.

We identified the 115 kDa protein by mass spectrometry (Figure S3B) and named it Giw1p (gentleman-in-waiting). The molecular weight of Giw1p as predicted from the *GIW1* mRNA sequence (GenBank XM_001029843) is 125 kDa. Giw1p shows no obvious similarity with any previously identified protein from any organism.

The interaction between Twi1p and Giw1p was confirmed by coimmunoprecipitation with the use of *GIW1*-HA strains in which all of the macronuclear *GIW1* loci were replaced by a *GIW1*-HA construct encoding C-terminal HA-tagged Giw1p (Figures S1I and S1J). *GIW1*-HA can replace essential function (see below) of *GIW1* in the production of sexual progeny (Figure S1M),

indicating that Giw1p-HA was functional and retained normal Giw1p physical interactions with other molecules. Two *GIW1*-HA or two nontagged strains were crossed, Giw1p-HA-containing complexes were immunoprecipitated with an anti-HA antibody, and the precipitated proteins were analyzed by Western blot with the use of an anti-Twi1p antibody. As shown in Figure 3B, a substantially higher amount of Twi1p was precipitated from the *GIW1*-HA strains than from the nontagged strains at all developmental stages tested, confirming that Twi1p and Giw1p are found in the same complex. Silver staining of these precipitated proteins detected only two specific proteins with the sizes of Giw1p-HA and Twi1p (Figure S3C), suggesting that Giw1p may complex only with Twi1p.

Giw1p Directly Binds to Twi1p

Because Twi1p associates with long noncoding RNAs (ncRNA) (Aronica et al., 2008), we determined whether the interaction between Twi1p and Giw1p was mediated by ncRNA. Lysates from *FLAG-HA-TWI1-WT* cells at 4 hr postmixing were incubated with 20 pg/mL of RNase A to degrade ncRNAs, and the Twi1p-Giw1p interaction was analyzed by immunoprecipitation with the use of an anti-FLAG antibody. The amount of Giw1p coimmunoprecipitated with FLAG-HA-Twi1p was comparable with (+) and without (−) RNaseA treatment (Figure 3C, Giw1p), whereas ncRNA was undetectable by RT-PCR in the immunoprecipitated sample from the RNase-treated lysate (Figure 3C, ncRNA RT+). These data suggest that the interaction between Twi1p and Giw1p is not mediated by long ncRNAs. This conclusion is further supported by the fact that Giw1p was coimmunoprecipitated with Twi1p from *EMA1* KO strains (Figure S3D), in which the Twi1p-ncRNA interaction is impaired (Aronica et al., 2008).

In the conditions described above, the amount of scnRNAs was unchanged after RNase A (20 pg/mL) treatment (+ in Figure 3C). However, scnRNAs were eliminated when we treated the lysate with a much higher concentration (100 μg/mL) of RNase A (++ in Figure 3C). Even in this condition, a significant, albeit reduced, amount (~60%) of Giw1p was coprecipitated with FLAG-HA-Twi1p (Figure 3C). This result suggests that Giw1p can interact with Twi1p in the absence of scnRNA in cell lysate. This conclusion is further supported by a GST pull-down assay using recombinant Twi1p expressed in *E. coli* and in vitro translated Giw1p. Giw1p was coprecipitated with full-length Twi1p fused with GST but not with GST alone (Figure 3D). Treatment with 100 μg/mL RNase A did not affect precipitation of Giw1p with GST-Twi1p (Figure 3D), suggesting that contaminating RNA does not mediate interaction of these two proteins. We conclude that Giw1p and Twi1p interact directly without RNA.

Twi1p shares conserved PAZ and Piwi domains with other Argonaute proteins (Mochizuki et al., 2002). To determine the domain(s) of Twi1p that interacts with Giw1p, we performed GST pull-down assays, using Giw1p and N-terminal, PAZ, Mid, Piwi, or C-terminal domains of Twi1p, each fused with GST. Giw1p coprecipitated with the PAZ and the Piwi domains but not with other domains of Twi1p (Figure 3D), indicating that Twi1p directly interacts with Giw1p through its PAZ and Piwi domains.

To determine which parts of Giw1p mediate the interaction with these domains, we divided Giw1p into six segments (Figure 3E), all of which we examined for binding with N-terminal, PAZ, Mid, or Piwi domains of Twi1p. Three of the six segments of Giw1p (N1, N3, and C2) were efficiently coprecipitated with PAZ and Piwi domains of Twi1p but were less efficiently coprecipitated with N-terminal and Mid domains (Figure 3E). Small amounts of the other three segments (N2, C1, and C3) were also coprecipitated with PAZ and Piwi domains, whereas a part of β-lactamase, which was used as a negative control, was not (Figure 3E), suggesting that these Giw1p segments also have binding activity, albeit weak, to PAZ and Piwi domains of Twi1p. Interaction between the N3 fragment of Giw1p and PAZ and Piwi domains of Twi1p was further confirmed by a reverse GST pull-down assay using GST-tagged Giw1p-N3 and His-tagged PAZ and Piwi domains (Figure S3E). His-PAZ and His-Piwi were coprecipitated with GST-Giw1p-N3 but not with GST alone. These results indicate that Giw1p has several different sites that have the ability to bind PAZ and Piwi domains of Twi1p and could bridge these domains.

Giw1p Is Specifically Expressed during Conjugation and Localizes to both the Cytoplasm and the Nuclei

Like *TWI1* mRNA expression (Mochizuki et al., 2002), *GIW1* mRNA expression occurs exclusively during early conjugation stages (2–4 hr postmixing) but was not detected in exponentially growing or starved vegetative cells (Figure 4A).

For study of the expression and localization of Giw1p, two *GIW1*-HA strains were crossed, and Giw1p-HA was detected with the use of an anti-HA antibody. Giw1p-HA was specifically detected during conjugation by Western blotting (Figure 4A). Indirect immunofluorescent staining showed that Giw1p-HA was localized to both the cytoplasm and the nuclei throughout conjugation (Figures 4B–4F).

Giw1p Is Required for Twi1p Nuclear Localization

To elucidate the function of Giw1p, we constructed *GIW1* knockout (KO) strains. All copies of the *GIW1* gene in the polyploid macronucleus were replaced by genes in which the entire coding sequence had been replaced by a drug-resistance marker (see Figures S1K and S1L).

Two wild-type or two *GIW1* KO strains were mated, and the localization of Twi1p was analyzed by indirect immunofluorescence staining with the use of an anti-Twi1p antibody. In wild-type cells, Twi1p was detected mainly in parental (Figures 5A and 5B) or newly developing (Figure 5C) macronuclei, whereas in *GIW1* KO cells, Twi1p localized to the cytoplasm throughout conjugation (Figures 5D–5F). These data indicate that Giw1p is required for nuclear localization of Twi1p.

We also analyzed the localization of Ema1p, Pdd1p, and Wag1p, which show localization patterns similar to those of Twi1p in wild-type cells (Figure S4). All of these proteins localize to macronuclei in *GIW1* KO cells (Figure S4), indicating that Giw1p is not a general nuclear transporter but is dedicated to Twi1p or to a limited set of proteins.

Giw1p Is Dispensable for Loading and Passenger-Strand Removal of scnRNAs

Given that Slicer-dead *TWI1* and *GIW1* KO strains showed a similar nuclear Twi1p localization defect, Giw1p could have a

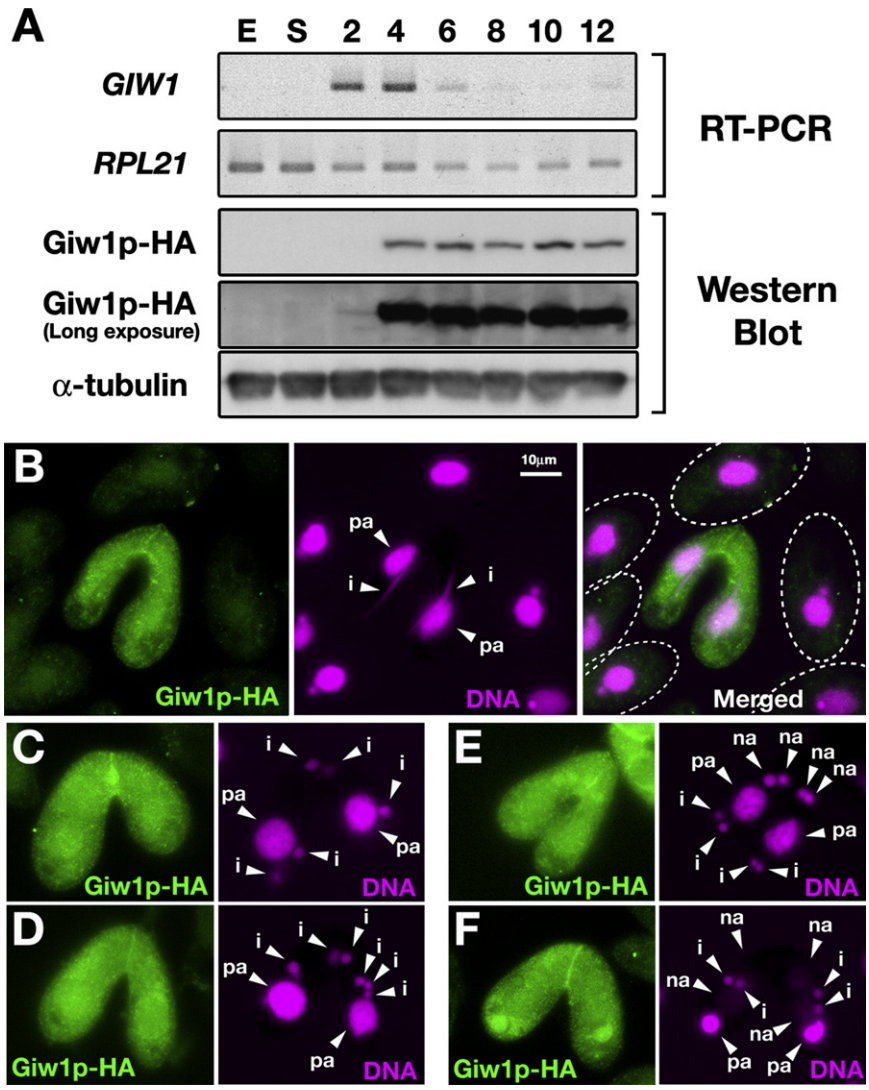

Figure 4. Giw1p Is Expressed Exclusively during Conjugation and Localizes to Both the Cytoplasm and the Nuclei

(A) Expression of *GIW1* mRNA and Giw1p-HA was analyzed by RT-PCR and Western blot, respectively. Total RNA of wild-type cells or total protein of *GIW1*-HA strains was extracted from exponentially growing vegetative (E), starved (S), or conjugating (2, 4, 6, 8, 10, and 12 hr postmixing) cells. Constitutively expressed *RPL21* mRNA and α-tubulin protein were also analyzed. Giw1p-HA and α-tubulin were detected by an anti-HA and an anti-α-tubulin antibody, respectively.

(B–F) *GIW1*-HA strains at early (B: meiotic prophase, C: meiosis), mid (D: pronuclear exchange), or late (E: macronuclear anlagen, F: nuclear alignment) stages of conjugation were fixed, and Giw1p-HA was localized by an anti-HA antibody (green). DNA was stained with DAPI (purple). In (B), nonmating cells (dotted lines) were included to show background staining. Micronuclei (i), parental macronuclei (pa), and new macronuclei (na) are marked.

function in passenger-strand removal of the Twi1p-associated scnRNAs. To assess this possibility, we immunoprecipitated Twi1p from wild-type and *GIW1* KO strains by using an anti-Twi1p antibody, and we analyzed Twi1p-associated scnRNA. Denaturing gel analysis indicated that similar amounts of scnRNA were associated with Twi1p in the presence and absence of Giw1p (Figure 6A). Native gel analysis detected little double-stranded scnRNA in the absence of *GIW1* (Figure 6B). We therefore conclude that Giw1p is not required for the production, loading, or passenger-strand removal of scnRNAs and that Giw1p most likely acts downstream of scnRNA passenger-strand removal in the pathway of Twi1p macronuclear import.

Presence of Double-Stranded scnRNA Inhibits Giw1p-Twi1p Interaction

For understanding of the relationship between the slicing of the scnRNA passenger strand and the action of Giw1p, two wild-type *FLAG-HA-TWI1-WT* strains or two Slicer-dead *FLAG-HA-TWI1-D526N* strains were mated, and FLAG-HA-Twi1p-WT or FLAG-HA-Twi1p-D526N were immunoprecipitated with the use of an anti-FLAG antibody. The coimmunoprecipitation of Giw1p was analyzed by Western blot with the use of an anti-Giw1p antibody. As shown in Figure 6C, Giw1p was coimmunoprecipitated with FLAG-HA-Twi1p-WT (WT), whereas no detectable Giw1p was precipitated with FLAG-HA-Twi1p-D526N (D526N). Similar results were obtained with the use of the other Slicer-dead mutant FLAG-HA-TWI1-H745Q (Figure S5). These results indicate that the Slicer activity of Twi1p has an essential role in the Twi1p-Giw1p interaction in vivo. Because Giw1p is required for the macronuclear localization of Twi1p (Figure 5), the lack of interaction between the Slicer-dead Twi1p mutants and Giw1p explains why Twi1p macronuclear localization is inhibited in the Slicer-dead *TWI1* strains. Because Slicer activity of Twi1p is important for the passenger-strand removal of scnRNAs, the inability of Slicer-dead Twi1p mutants to interact with Giw1p in vivo is likely caused by the association of double-stranded scnRNAs with these mutants.

To test this hypothesis, we analyzed the effect of double-stranded scnRNAs on Twi1p-Giw1p interaction in vitro (Figure 6D). Recombinant GST alone (lane 1) or wild-type Twi1p fused to GST (GST-Twi1p) (lanes 2–4) was first incubated with (lanes 3 and 4) or without (lanes 1 and 2) a 28 nt guide RNA, then with (lane 4) or without (lanes 1–3) a phosphorothioate-modified noncleavable 28 nt target (passenger) RNA. Then, GST-pull down assays were performed with the radiolabeled Giw1p. The amount of Giw1p coprecipitated with GST-Twi1p was greatly reduced when GST-Twi1p was preincubated with a guide and a noncleavable target RNA (lane 4), but not

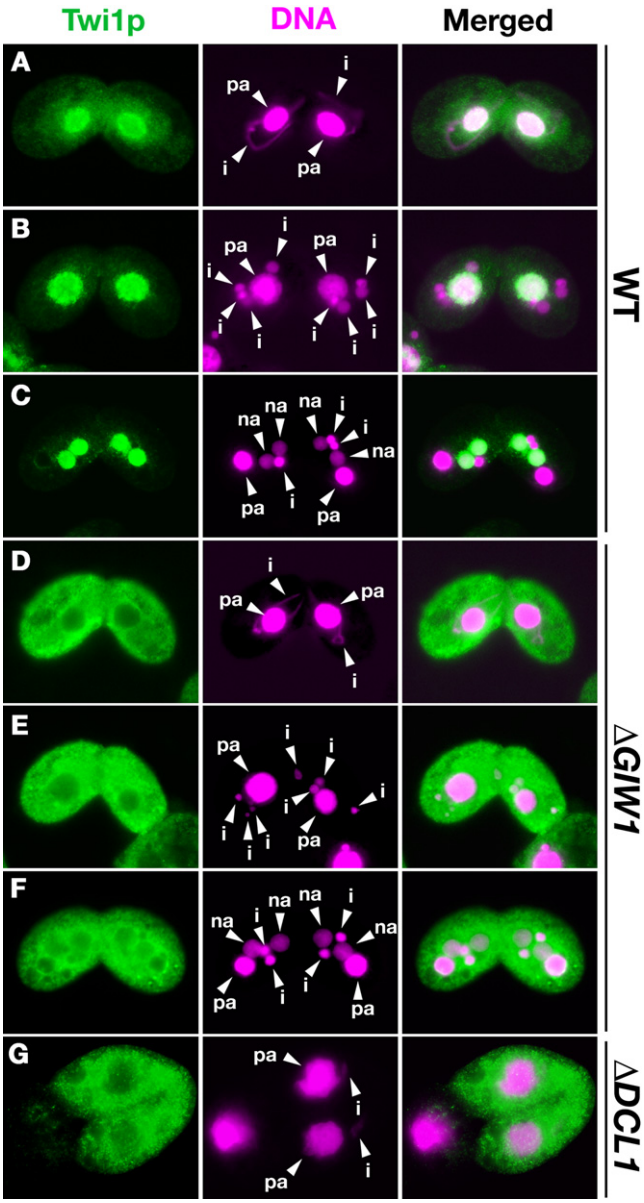

Figure 5. Twi1p Is Mislocalized to the Cytoplasm in the Absence of *GIW1*

Localization of Twi1p in wild-type (WT), *GIW1* KO (Δ*GIW1*), and *DCL1* KO (*ΔDCL1*) strains at early (meiotic prophase; A, D, and G), mid (postzygotic mitosis; B and E), and late (nuclear alignment; C and F) stages of conjugation was analyzed with the use of an anti-Twi1p antibody (green). DNA was stained by DAPI (purple). Micronuclei (i), parental macronuclei (pa), and new macronuclei (na) are marked with arrowheads.

See also Figure S4.

with a guide RNA alone (lane 3), indicating that the presence of double-stranded scnRNAs in Twi1p inhibits the Giw1p-Twi1p interaction. These results suggest that Giw1p can sense the state of the scnRNA complexed with Twi1p and binds to both unloaded Twi1p and Twi1p that is associated with single-stranded scnRNA.

Dicer-like Protein Is Required for Nuclear Localization of Twi1p

Given that Giw1p can bind to Twi1p without scnRNA in a cell lysate (Figure 3C) and in vitro (Figure 3D), it seems reasonable to expect that unloaded Twi1p could localize in the macronucleus. However, Twi1p was localized to the cytoplasm in the *DCL1* KO cells (Figure 5G), in which no detectable scnRNAs are produced (Malone et al., 2005; Mochizuki and Gorovsky, 2005). One possibility is that loaded single-stranded scnRNA may be required for binding to the nuclear import machinery. Alternatively, given that a significant proportion of Twi1p that enters into the macronucleus in a wild-type cell is predicted to be released from scnRNA by the selective degradation of scnRNAs complementary to the macronuclear DNA (Mochizuki and Gorovsky, 2004), there may be a mechanism that exports unloaded Twi1p to the cytoplasm, where Twi1p could load a new scnRNA cargo, thereby preventing accumulation of unloaded Twi1p in the nucleus.

Twi1p-Slicer and Giw1p Are Required for DNA Elimination

In both the Slicer-dead *TWI1* and *GIW1* KO strains, Twi1p is not localized to the developing macronucleus (Figures 2G and 2H, Figure 5F), where the Twi1p-scnRNA complexes are required for DNA elimination. We studied DNA elimination at four different loci by single-progeny PCR (Figure 7A) and found that their eliminations were indeed inhibited in the progeny of Slicer-dead *FLAG-HA-TWI1-D526N* strains (Figure 7B).

Because most of the *GIW1* KO cells are blocked midconjugation (see below), it was difficult to study their DNA elimination by PCR. Instead, we used fluorescence in situ hybridization (FISH) to analyze DNA elimination of the Tlr1 and the REP IES elements, which are moderately repeated in the micronuclear genome (Wuitschick et al., 2002; Fillingham et al., 2004). Both elements remained present in the new macronucleus of most of the progeny of *GIW1* KO, as well as of *FLAG-HA-TWI1-D526N* strains at 36 hr postmixing, but were completely removed in the progeny of wild-type cells (Figure 7C). Therefore, the absence of Giw1p inhibits DNA elimination of these IES elements.

Like all other known mutants showing defective DNA elimination, Slicer-dead *TWI1* and *GIW1* KO strains did not produce viable mating progeny (Figure S1M). In addition, *GIW1* KO strains showed developmental arrest, and ~70% of cells aborted mating midconjugation (Figure S6). This phenotype was not observed in *TWI1* KO strains (Mochizuki and Gorovsky, 2004) and Slicer-dead *TWI1* mutants (data not shown). Given that scnRNAs are believed to be derived from genic as well as nongenic sequences, they potentially target many different mRNAs for degradation if they are not properly regulated. Thus, the pleiotropic defects in *GIW1* KO cells could be due to the presence of mature Twi1p-scnRNA complexes in the cytoplasm. Alternatively, because Giw1p binds to the PAZ and Piwi domains of Twi1p (Figures 3D and 3E), Giw1p might block Twi1p-associated scnRNAs from binding to mRNAs (or other RNAs) or directly inhibit Slicer activity of Twi1p.

DISCUSSION

In this study, we have shown that the *Tetrahymena* Argonaute protein Twi1p has Slicer activity and that this activity is essential

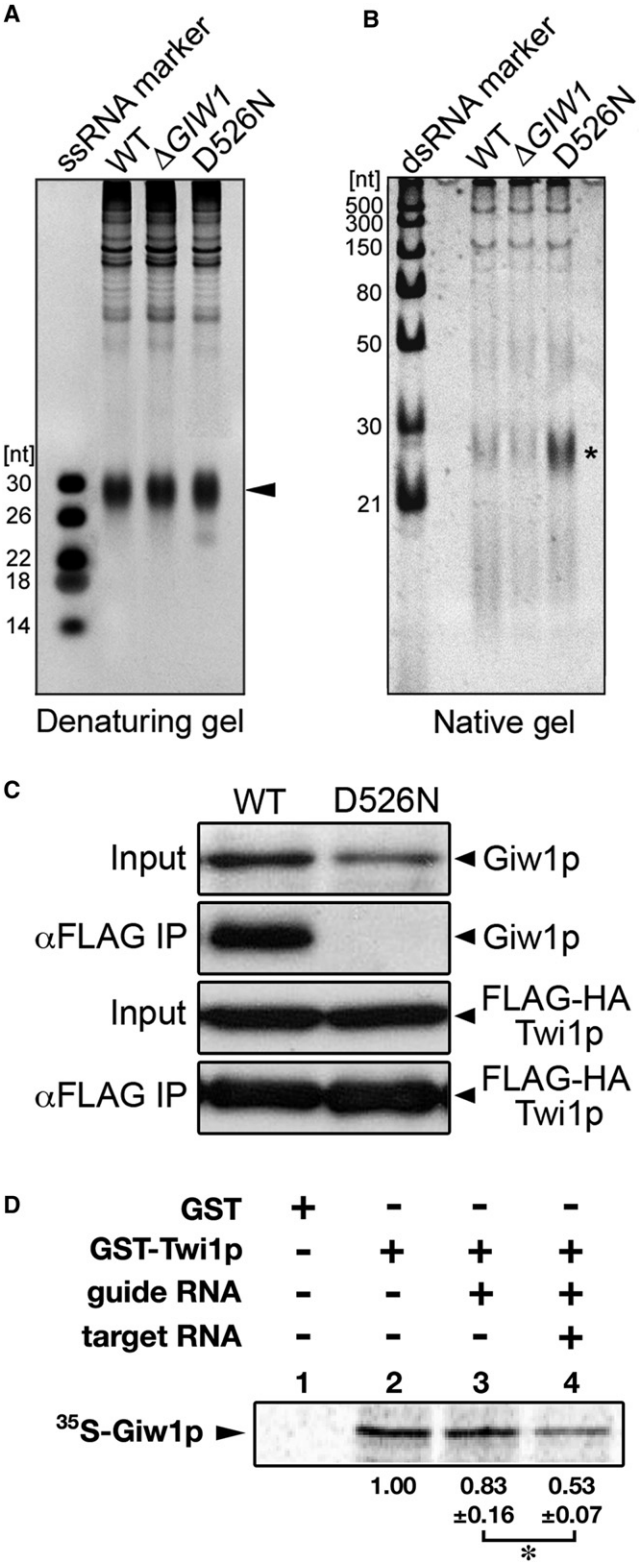

Figure 6. Giw1p Binds to Twi1p Complexed with Single-Stranded scnRNA

(A and B) Twi1p-containing complexes from wild-type (WT), *GIW1* knockout (Δ*GIW1*), and *FLAG-HA-TWI1-D526N* (D526N) strains at 4 hr postmixing were immunoprecipitated with the use of an anti-Twi1p antibody. Coprecipitated RNA was separated in a denaturing (A) or in a native (B) gel and stained by GelRed. Single-stranded (ss) or double-stranded (ds) RNA markers were included. scnRNA in the denaturing gel is marked with an arrowhead. Double-stranded scnRNA in the native gel is marked with an asterisk.

(C) Cell lysate (input) was prepared from FLAG-HA-TWI1-WT (WT) or FLAG-HA-TWI1-D526N (D526N) strains at 4 hr postmixing, and FLAG-HA-Twi1p-containing complexes were immunoprecipitated with the use of an anti-FLAG antibody (α-FLAG IP). Giw1p and FLAG-HA-Twi1p were detected by Western blot with the use of anti-Giw1p and anti-FLAG antibodies, respectively.

(D) GST pull-down assays were performed with the use of GST (lane 1) or GST-Twi1p (lanes 2–4), and ^{35}S-labeled Giw1p (^{35}S-Giw1p). In the experiment shown in lanes 3 and 4, GST-Twi1p was first incubated with 28 nt guide-strand RNA, then with (lane 4) or without (lane 3) 28 nt noncleavable target RNA that was complementary to the guide-strand RNA prior to the incubation with ^{35}S-Giw1p. Precipitated proteins were separated by SDS-PAGE, and ^{35}S-Giw1p was detected by phosphorimager. Relative amounts of ^{35}S-Giw1p detected (average ± standard deviation from four experiments) are shown at the bottom. Asterisk (*) indicates p = 0.014.

See also Figure S5.

for its macronuclear localization. We have also identified a Twi1p-associated protein, Giw1p, which is required for macronuclear localization of Twi1p. Giw1p binds to wild-type Twi1p, but not to Slicer-dead Twi1p, in vivo. These results indicate that Slicer-dependent passenger-strand removal of scnRNAs is a prerequisite for the Twi1p-Giw1p interaction, which, in turn, is essential for the macronuclear localization of Twi1p. Thus, Giw1p serves as a gatekeeper that allows only mature Twi1p-scnRNA complexes to enter macronuclei. Currently, the detailed mechanism by which Giw1p functions is not clear. The simplest hypothesis is that Giw1p might be an adaptor protein that connects the Twi1p-scnRNA complex to nuclear import machinery.

Given that the nuclear localization of some Argonaute proteins is dependent on the presence of their small RNA cargos in nematodes (Guang et al., 2008) and in mice (Aravin et al., 2008), small RNA-dependent nuclear localization of Argonaute proteins is probably widespread among eukaryotes. This study reveals yet another layer of the regulatory mechanisms for the nuclear localization of small RNA-Argonaute complexes: the requirement for passenger-strand removal for the nuclear import of a small RNA-Argonaute complex. This mechanism might have evolved to provide Argonaute proteins enough time to release aberrant RNAs and to find correct RNAs before they are imported into the nucleus. Alternatively, proteins that block or modulate the activity of mature small RNA-Argonaute complexes during their transport might have evolved first and then may have later acquired a direct role in the nuclear import process. Because it is not yet known whether maturation of Argonaute-small RNA complexes is required for nuclear transport of Argonaute proteins in other eukaryotes, the localization of Slicer-dead Argonautes will be of interest to study in other systems.

The conformation of a bacterial Argonaute protein changes according to the state of nucleic acids with which the protein is complexed, such that the space between the PAZ and Piwi domains is wider when it is associated with both guide and substrate strands than when it is associated with only a guide

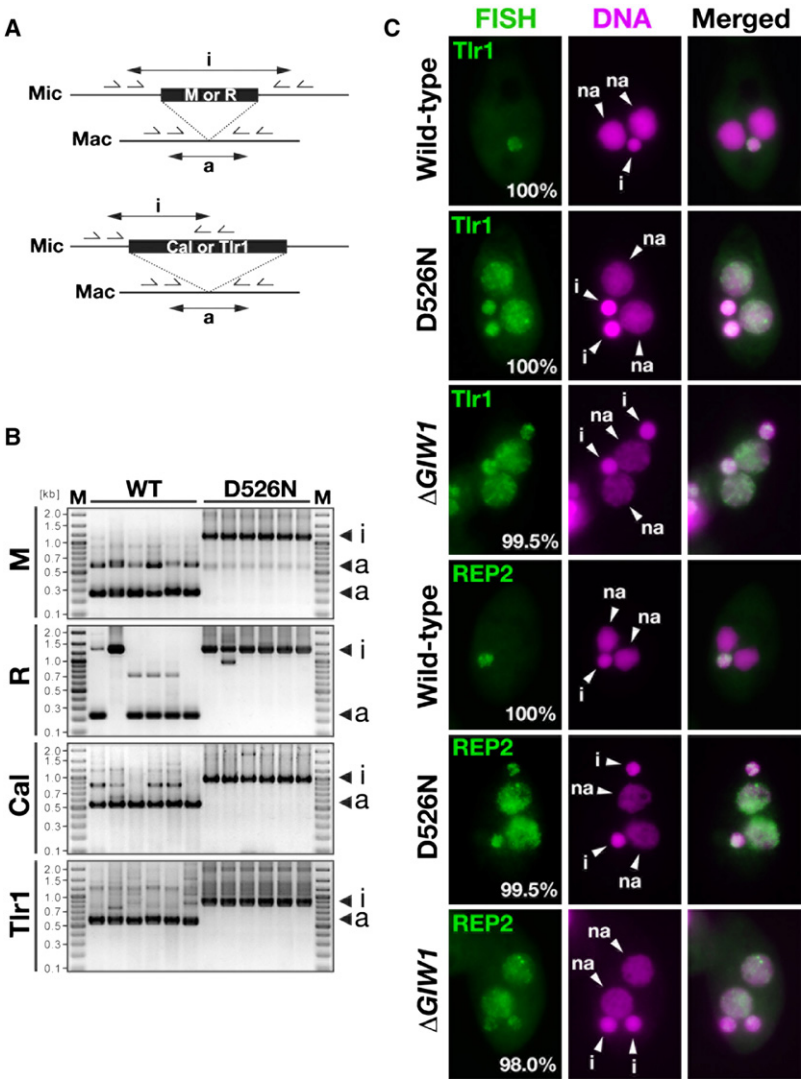

Figure 7. DNA Elimination Is Inhibited in the Progeny of Slicer-Dead *TWI1* and *GIW1* KO Cells

(A and B) DNA-elimination assays by PCR. (A) Horizontal lines, filled boxes, and arrows indicate macronuclear-destined sequences, eliminated DNAs (IESs), and primers for nested PCR, respectively. (B) Single exconjugants (sexual progeny) from FLAG-HA-*TWI1*-WT (WT) and FLAG-HA-*TWI1*-D526N (D526N) were assayed. The sizes of the unprocessed (micronuclear form) and the processed (macronuclear form) products are marked with "i" and "a," respectively.

(C) Exconjugants of wild-type (WT), Slicer-dead FLAG-HA-TWI1-D526N (D526N), and *GIW1* KO (Δ*GIW1*) at 36 hr post mixing were used for detecting Tlr1- and REP-IES elements by FISH (green). DNA was stained with DAPI (purple). The micronuclei (i) and the new macronuclei (na) are marked. The percentage of total exconjugants that showed phenotypes represented in the pictures is given (n = 200).

See also Figure S6.

strand (Wang et al., 2008). Because Giw1p binds to both the PAZ and the Piwi domains of Twi1p (Figure 3D), we propose that scnRNA passenger-strand removal also alters the distance between the PAZ and Piwi domains, allowing binding of Giw1p. Given that Giw1p is the only currently known protein that can detect the state of small RNAs (double or single stranded) associated with Argonaute proteins, identification of functional homologs of Giw1p in other eukaryotes could aid in understanding how conformational changes of Argonaute proteins affect their functions. Because Giw1p shows no obvious similarity with any previously identified proteins, determination of its crystal structure should prove valuable in identifying such homologs and in elucidating mechanisms of this process.

EXPERIMENTAL PROCEDURES

General Methods and Oligonucleotides

Tetrahymena strains, culture conditions, DNA-elimination assay, progeny-viability assay, and oligonucleotide used are described in the Extended Experimental Procedures.

Production of Recombinant Proteins

GST- or His-tagged Twi1p, hAgo1, and Giw1p were expressed in *E. coli*. ^{35}S-labeled full-length and partial Giw1p were synthesized by an in vitro translation system. See the Extended Experimental Procedures for the detailed procedures.

Slicer Assay

Approximately 3 pmol of the recombinant GST or GST fusion proteins were preincubated with 3 pmol of 27-mer or 21-mer guide RNAs (27-mer for Twi1p and 21-mer for hAgo2) in 30 μl of 1× cleavage buffer [30 mM HEPES (pH 7.4), 40 mM KOAc, 5 mM $Mg(OAc)_2$, 5 mM DTT] containing 1 μg BSA, 0.5 μg yeast RNA (Ambion), and 40 U RNasin (Promega) for 90 min at 25°C (for Twi1p) or 37°C (for hAgo2). 27-mer (for Twi1p) or 21-mer (for hAgo2) ^{32}P-labeled Target RNA was added and incubated for 90 min at 25°C (for Twi1p) or 37°C (for hAgo2). RNA was extracted with phenol-chloroform followed by ethanol precipitation, separated in a 20% denaturing polyacrylamide gel, and analyzed by autoradiography.

Antibody Production

Rabbit anti-Giw1p and anti-Wag1p antibodies were produced with the use of synthetic peptides. See the Extended Experimental Procedures for the detailed procedures.

Coimmunoprecipitation

Cells (2×10^6 in total) were lysed by sonication in 1 ml lysis buffer A [20 mM Tris pH 7.5, 100mM NaCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 1% Tween 20, 0.1 mM PMSF, 1× complete protease inhibitor cocktail (Roche), and 0.4 U/ml RNasin (Promega)]. For Giw1p-HA immunoprecipitation, lysis buffer B (buffer A without Tween 20) was used. FLAG-HA-Twi1p, Giw1p-HA, or Twi1p complex was immunoprecipitated with the use of anti-FLAG (M2, Sigma), anti-HA (HA-7, Sigma) or anti-Twi1p (Aronica et al., 2008) antibody, respectively. FLAG-HA-Twi1p complexes were eluted in 0.3 mg/ml 3× FLAG peptide (Sigma). RNA in the eluate was extracted by TRIzol. Giw1p-HA and Twi1p complexes were eluted by boiling the gels in SDS-PAGE sample buffer or by incubating gels in the TRIzol. RNAs were separated in 15% denaturing gels or on 18% native polyacrylamide gels and were detected either directly by GelRed (Gentauer) or by northern blot (Aronica et al., 2008) probed with 5′-end-radiolabeled

oligo DNAs (M-28nt, Tlr1-28nt, or Tlr1-1). ncRNA was analyzed by RT-PCR (Aronica et al., 2008). FLAG-HA-Twi1p, Giw1p, and Twi1p were detected by Western blot with the use of anti-FLAG, anti-Giw1p, and anti-Twi1p antibodies, respectively.

Immunofluorescence Staining

Cells were fixed and processed as described previously (Loidl and Scherthan, 2004). See the Extended Experimental Procedures for the detailed procedures.

Nuclear-Cytoplasmic Fractionation

A pellet of 2×10^6 cells was gently resuspended in the ice-cold 1 ml lysis buffer [10 mM Tris pH 7.5, 5 mM $MgCl_2$, 10 mM KCl, 0.05% Triton X-100, 1× complete protease inhibitor cocktail (Roche)] and immediately centrifuged at 3,000 rpm for 5 min at 4°C. The supernatant was mixed with an equal volume of 2x SDS-PAGE sample buffer. The pellet was resuspended with the lysis buffer to make final volume 1 ml and was mixed with an equal volume of 2x SDS-PAGE sample buffer.

Identification of Giw1p

FLAG-HA-*TWI1* strains at 5 or 9 hr postmixing were lysed with a Dounce homogenizer in lysis buffer B, proteins were immunoprecipitated with anti-HA (HA-7) agarose, and eluted with 0.2 mg/mL HA peptide (Sigma). The eluate was separated by SDS-PAGE and visualized by silver or Coomassie Blue staining. The Coomassie Blue-stained ~115 kDa band was analyzed as described (Bowman et al., 2005).

GST-Pull Down Assay

For the experiment shown in Figures 3D and 3E, GST, GST-Twi1p, or GST-Giw1p-N3 (~1 μg) was incubated with 20 μl glutathione sepharose 4B resin (GE Healthcare) in GST pull-down buffer (GPB) [20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.1% BSA] for 30 min at 4°C. For the experiment shown in Figure 6D, GST or GST-Twi1p (~2 μg) in PBS with 5 mM $Mg(OAc)_2$, 5 mM DTT, and ribolock RNase inhibitor (Fermentas) were incubated with or without 4.4 nmol 28 nt guide RNA for 90 min at 26°C. Then, 17–22 nmol of 28 nt noncleavable target RNA, 24 nt or 28 nt nontarget RNA (both provided similar results), or water was added and the reaction was incubated for 90 min at 26°C. A total of 20 μl glutathione sepharose 4B resin in GPB was added, and the reactions were incubated for 30 min at 4°C. The beads were washed with GPB and incubated with ^{35}S-labeled full-length or partial Giw1p recombinant protein (1.2 to 2 μl reaction of in vitro translation) or with His-tagged PAZ, mid, or Piwi domain of Twi1p (~0.4 μg) in GPB for 90–120 min at 4°C. The beads were washed with GPB and boiled in SDS-PAGE sample buffer, and the elutions were separated by SDS-PAGE. ^{35}S-labeled proteins were detected by phosphorimager (GE Healthcare) or by autoradiography. His-tagged proteins were detected by Western blot with the use of an anti-His antibody (QIAGEN).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and six figures and can be found with this article online at doi:10.1016/j.cell.2010.02.010.

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